PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

Commissioner
US Department of Commerce
United States Patent and Trademark
Office, PCT
2011 South Clark Place Room
CP2/5C24
Arlington, VA 22202
ETATS-UNIS D'AMERIQUE

in its capacity as elected Office

Date of mailing (day/month/year) 12 February 2001 (12.02.01)	
International application No. PCT/PT00/00004	**Applicant's or agent's file reference** 84 810 - PCT
International filing date (day/month/year) 31 May 2000 (31.05.00)	**Priority date** (day/month/year) 31 May 1999 (31.05.99)
Applicant LEÃO, Cecília et al	

1. The designated Office is hereby notified of its election made:

[X] in the demand filed with the International Preliminary Examining Authority on:

15 December 2000 (15.12.00)

[ ] in a notice effecting later election filed with the International Bureau on:

2. The election [X] was

[ ] was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO **34, chemin des Colombettes** **1211 Geneva 20, Switzerland**	Authorized officer Juan Cruz
Facsimile No.: (41-22) 740.14.35	Telephone No.: (41-22) 338.83.38

Copy from the Elected Office (EO/SO)

PATENT COOPERATION TREATY

PCT

NOTIFICATION OF THE RECORDING OF A CHANGE

(PCT Rule 92bis.1 and Administrative Instructions, Section 422)

From the INTERNATIONAL BUREAU

To:

FERREIRA MAGNO, Fernando, António,
Rua das Flores, 74 - 4º andar
P-1200-195 Lisboa
PORTUGAL

Date of mailing (day/month/year)
16 November 2001 (16.11.01)

IMPORTANT NOTIFICATION

Applicant's or agent's file reference
84 810 - PCT

International application No.
PCT/PT00/00004

International filing date (day/month/year)
31 May 2000 (31.05.00)

1. The following indications appeared on record concerning:

[X] the applicant [ ] the inventor [ ] the agent [ ] the common representative

Name and Address
STAB-TRATAMENTO DE ÁGUAS E BIOTECNOLOGIA, LDA.
Quinta de Stª. Teresa
P-2825 Charneca da Caparica
Portugal

State of Nationality: PT
State of Residence: PT
Telephone No.:
Facsimile No.:
Teleprinter No.:

2. The International Bureau hereby notifies the applicant that the following change has been recorded concerning:

[X] the person [X] the name [X] the address [ ] the nationality [ ] the residence

Name and Address
STAB VIDA - INVESTIGACÃO E SERVIÇOS EM CIÊNCIAS BIOLÓGICAS, LDA.
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2781-601 Oeiras
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State of Nationality: PT
State of Residence: PT
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Facsimile No.:
Teleprinter No.:

3. Further observations, if necessary:

4. A copy of this notification has been sent to:

[X] the receiving Office
[ ] the International Searching Authority
[ ] the International Preliminary Examining Authority
[ ] the designated Offices concerned
[X] the elected Offices concerned
[ ] other:

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland
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Authorized officer
Dominique DELMAS
Telephone No.: (41-22) 338.83.38

PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION CONCERNING SUBMISSION OR TRANSMITTAL OF PRIORITY DOCUMENT

(PCT Administrative Instructions, Section 411)

To:

FERREIRA MAGNO, Fernando, António,
Rua das Flores, 74 - 4º andar
P-1200-195 Lisboa
PORTUGAL

Date of mailing (day/month/year) 05 July 2000 (05.07.00)	
Applicant's or agent's file reference 84 810 - PCT	**IMPORTANT NOTIFICATION**
International application No. PCT/PT00/00004	International filing date (day/month/year) 31 May 2000 (31.05.00)
International publication date (day/month/year) Not yet published	Priority date (day/month/year) 31 May 1999 (31.05.99)
Applicant UNIVERSIDADE DO MINHO et al	

1. The applicant is hereby notified of the date of receipt (except where the letters "NR" appear in the right-hand column) by the International Bureau of the priority document(s) relating to the earlier application(s) indicated below. Unless otherwise indicated by an asterisk appearing next to a date of receipt, or by the letters "NR", in the right-hand column, the priority document concerned was submitted or transmitted to the International Bureau in compliance with Rule 17.1(a) or (b).

2. This updates and replaces any previously issued notification concerning submission or transmittal of priority documents.

3. An asterisk(*) appearing next to a date of receipt, in the right-hand column, denotes a priority document submitted or transmitted to the International Bureau but not in compliance with Rule 17.1(a) or (b). In such a case, the attention of the applicant is directed to Rule 17.1(c) which provides that no designated Office may disregard the priority claim concerned before giving the applicant an opportunity, upon entry into the national phase, to furnish the priority document within a time limit which is reasonable under the circumstances.

4. The letters "NR" appearing in the right-hand column denote a priority document which was not received by the International Bureau or which the applicant did not request the receiving Office to prepare and transmit to the International Bureau, as provided by Rule 17.1(a) or (b), respectively. In such a case, the attention of the applicant is directed to Rule 17.1(c) which provides that no designated Office may disregard the priority claim concerned before giving the applicant an opportunity, upon entry into the national phase, to furnish the priority document within a time limit which is reasonable under the circumstances.

Priority date	Priority application No.	Country or regional Office or PCT receiving Office	Date of receipt of priority document
31 May 1999 (31.05.99)	102305	PT	20 June 2000 (20.06.00)

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland

Facsimile N . (41-22) 740.14.35

Authorized officer

Peggy Steunenberg

Telephone N . (41-22) 338.83.38

WO 00/73494
PCT/PT00/00004

PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTICE INFORMING THE APPLICANT OF THE COMMUNICATION OF THE INTERNATIONAL APPLICATION TO THE DESIGNATED OFFICES

(PCT Rule 47.1(c), first sentence)

To:
FERREIRA MAGNO, Fernando, António,
Rua das Flores, 74 - 4º andar
P-1200-195 Lisboa
PORTUGAL

Date of mailing (day/month/year) 07 December 2000 (07.12.00)	
Applicant's or agent's file reference 84 810 - PCT	**IMPORTANT NOTICE**

International application No.	**International filing date** (day/month/year)	**Priority date** (day/month/year)
PCT/PT00/00004	31 May 2000 (31.05.00)	31 May 1999 (31.05.99)

Applicant
UNIVERSIDADE DO MINHO et al

1. Notice is hereby given that the International Bureau has communicated, as provided in Article 20, the international application to the following designated Offices on the date indicated above as the date of mailing of this Notice:
   AG,AU,DZ,KP,KR,US

   In accordance with Rule 47.1(c), third sentence, those Offices will accept the present Notice as conclusive evidence that the communication of the international application has duly taken place on the date of mailing indicated above and no copy of the international application is required to be furnished by the applicant to the designated Office(s).

2. The following designated Offices have waived the requirement for such a communication at this time:
   AE,AL,AM,AP,AT,AZ,BA,BB,BG,BR,BY,CA,CH,CN,CR,CU,CZ,DE,DK,DM,EA,EE,EP,ES,FI,GB,GD,
   GE,GH,GM,HR,HU,ID,IL,IN,IS,JP,KE,KG,KZ,LC,LK,LR,LS,LT,LU,LV,MA,MD,MG,MK,MN,MW,MX,
   NO,NZ,OA,PL,PT,RO,RU,SD,SE,SG,SI,SK,SL,TJ,TM,TR,TT,TZ,UA,UG,UZ,VN,YU,ZA,ZW
   The communication will be made to those Offices only upon their request. Furthermore, those Offices do not require the applicant to furnish a copy of the international application (Rule 49.1(a-bis)).

3. Enclosed with this Notice is a copy of the international application as published by the International Bureau on
   07 December 2000 (07.12.00) under No. WO 00/73494

REMINDER REGARDING CHAPTER II (Article 31(2)(a) and Rule 54.2)

If the applicant wishes to postpone entry into the national phase until 30 months (or later in some Offices) from the priority date, a **demand for international preliminary examination** must be filed with the competent International Preliminary Examining Authority before the expiration of 19 months from the priority date.

It is the applicant's sole responsibility to monitor the 19-month time limit.

Note that only an applicant who is a national or resident of a PCT Contracting State which is bound by Chapter II has the right to file a demand for international preliminary examination.

REMINDER REGARDING ENTRY INTO THE NATIONAL PHASE (Article 22 or 39(1))

If the applicant wishes to proceed with the international application in the **national phase**, he must, within 20 months or 30 months, or later in some Offices, perform the acts referred to therein before each designated or elected Office.

For further important information on the time limits and acts to be performed for entering the national phase, see the Annex to Form PCT/IB/301 (Notification of Receipt of Record Copy) and Volume II of the PCT Applicant's Guide.

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland

Facsimile No. (41-22) 740.14.35

Authorized officer
J. Zahra

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PATENT COOPERATION TREATY

PCT

REC'D 24 APR 2001
WIPO PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
84 810 - PCT		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/PT00/00004	31/05/2000	31/05/1999
International Patent Classification (IPC) or national classification and IPC		
C12Q1/04		
Applicant		
UNIVERSIDADE DO MINHO et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 7 sheets, including this cover sheet.

   ☒ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 16 sheets.

CORRECTED VERSION

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☐ Priority
   - III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☐ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   - VI ☐ Certain documents cited
   - VII ☐ Certain defects in the international application
   - VIII ☒ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
15/12/2000	20.04.2001
Name and mailing address of the international preliminary examining authority:	Authorized officer
European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Herrero, M Telephone No. +49 89 2399 8542

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/PT00/00004

I. Basis of th report

1. With regard to the **elements** of the international application *(Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17))*:
Description, pages:

3-5,12,17,18,20	as originally filed
1,2,6-11,13-15,15a, 19	filed with the demand

Claims, No.:

12 (part),13-16	as originally filed
1-11,12 (part)	filed with the demand

Drawings, sheets:

1/2,2/2	as originally filed

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

   These elements were available or furnished to this Authority in the following language: , which is:

   - ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
   - ☐ the language of publication of the international application (under Rule 48.3(b)).
   - ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

   - ☐ contained in the international application in written form.
   - ☐ filed together with the international application in computer readable form.
   - ☐ furnished subsequently to this Authority in written form.
   - ☐ furnished subsequently to this Authority in computer readable form.
   - ☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
   - ☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

   ☐ the description, pages:

   ☐ the claims, Nos.:

   ☐ the drawings, sheets:

5. ☒ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

   (Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)
   see separate sheet

6. Additional observations, if necessary:
   see separate sheet

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	1-16
	No:	Claims	
Inventive step (IS)	Yes:	Claims	1-16
	No:	Claims	
Industrial applicability (IA)	Yes:	Claims	1-16
	No:	Claims	

2. Citations and explanations
   see separate sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:
see separate sheet

SECTION I

5. The amendments filed with the demand (which have been explained in the Applicant's letter of 21.03.01) introduce subject-matter which extends beyond the content of the application as filed, contrary to Article 34(2)(b) PCT. The amendments concerned are the following (underlined):

On page 10, Table 3; page 11, Table 4 and page 13, Table 5, a line has been inserted between the 3rd and 4th lines in which the number of tested strains for the species *Zygosaccharomyces rouxii* is identified as **6**.

The explanations given by the Applicants with respect to the origin of the proposed amendments have been taken into account. Thus the present Examining Authority is of the opinion that a basis for the introduction of an apparent missing line in the aforementioned positions of Tables 3, 4 and 5, referring to **one (1)** strain of the species *Zygosaccharomyces rouxii* having being tested and displaying a negative result, whereby the culture medium maintained its green color, is indeed found in the original application, e.g. in view of page 6, lines 8-9 and Figure 2, on page 1/2 of the drawings (cf *Z. rouxii* IGC 4194).

Nevertheless, no basis could be found in the application as originally filed for the proposed amendment indicating that in the case of *Zygosaccharomyces rouxii* the number of tested strains was **6**.

Summarizing, support for the maintenance of the *Zygosaccharomyces rouxii* in the Tables 3, 4 and 5 as one of the tested strains for which a negative result was obtained (i.e. the medium remained green) is indeed found in the original application. Accordingly, this report has been established as if the amendment carried out on page 10, Table 3; page 11, Table 4 and page 13, Table 5 (i.e. the line inserted between the 3rd and 4th lines) would have stated:

"*Zygosaccharomyces rouxii* 1 green"

6. Additional observations

Present Claims 1 to 12(part), filed with the demand, embrace amendments to original Claims 5 and 10 carried out under Art. 34 PCT, as well as amendments under Art. 19 PCT to original Claims 3, 5 and 10, which were filed with the letter dated 06.11.00.

SECTION V

2. CITATIONS AND EXPLANATIONS

2.1 The present application discloses a differential and selective culture medium suitable for the detection and identification of *Zygosaccharomyces bailii* and *Zygosaccharomyces bisporus* yeasts, which is considered to be neither anticipated nor suggested by the documents cited in the International Search Report. The novel selective medium, which seems to be specially useful for detecting the aforementioned food deteriorating yeasts, comprises a base mineral medium supplemented with vitamins, oligoelements, glucose and formic acid as the only carbon and energy sources, and bromocresol green as an acid-base indicator having a pK_i between 4.5 and 4.8. This indicator provides the medium with a green color, that is converted to blue through the action of *Z. bailii* and *Z. bisporus* yeasts during incubation under appropriate conditions. As substantiated in the description (see Examples 1 and 2), when growing on solid medium, the colonies formed by *Z. bailii* and *Z. bisporus* will be easily distinguishable from colonies arising from other possible yeasts, because they present a blue color.

In line with the above present Claims 1-16 would appear to relate to subject-matter which is novel and inventive over the available prior art and thus satisfies the criteria set forth in Art. 33(2) and (3) PCT (however, see Section VIII below).

The subject-matter encompassed by Claims 1 to 16 is also susceptible of industrial applicability as required by Art. 33(4) PCT

SECTION VIII

1. It is clear from the description on page 3, lines 13-18 that the use of an acid-base indicator having a pK_i between 4.5 and 4.8 (particularly bromocresol green) is a feature essential to the performance of the invention.

Since independent Claims 1 and 12 do not contain this feature they do not meet the requirement following from Article 6 PCT taken in combination with Rule 6.3(b) PCT that any independent claim must contain all the technical features essential to the definition of the invention.

In connection with the foregoing objection it is noted that no appropriate acid-base indicator different from bromocresol green is mentioned in the present disclosure (see the supporting description on page 3, lines 15-17; page 4, lines 7-11; page 7, lines 13-14 and 19-26; Table 1 on page 8 and the Examples). Independent Claims

1 and 12 are therefore not supported by the description as required by Article 6 PCT, as their scope is broader than justified by the actual disclosure of the invention.

The above deficiency affects *mutatis mutandis* the definition of the intended subject-matter according to dependent Claims 2-5, 8-9, 11 and 14 and appended Claims 15 and 16, insofar as these claims rely either on the medium of Claim 1 or the process of Claim 12.

2. On page 4, lines 17-22 of the description the following is explained:

"The culture medium object of the invention is prepared by autoclave sterilization of the base mineral medium in deionized water. The medium is then allowed to cool, and before solidifying, the glucose, formic acid, oligoelements and vitamins, prepared as adequate solutions and previously sterilized, are added under aseptic conditions"

Considering the foregoing, the term "mixed", which is employed on present page 9, line 10 (instead of the previously used "annealed") seems to be inadequate. Probably the subject sentence instead of "... are *annealed at* ...", was meant to read, for instance, "The solution and the base medium are *brought to* ..."

It is also noted that present Claim 11 reads "... *annealing* this solution and the base medium at..." (but in line with the above should have better indicated, for example, "*... bringing* this solution and the base medium *to*...).

Moreover, it seems that the last line of Claim 11 should have read "before mixing the same and adjusting the final pH to the desired value"

3. The statement in the description on page 20, lines 13-16 implies that the subject-matter for which protection is sought may be different to that defined by the claims, thereby resulting in lack of clarity (Article 6 PCT) when used to interpret them (see also the PCT Guidelines, III-4.3a).

4. The following expressions appear to contain wording/clerical mistakes:

"... the filters are then placed Petri dishes..." (page 14, lines 3-4).

"... acid formic..." (page 18, line 9).

"... culture medium for *Zygosaccharomyces bailii* e *Zygosaccharomyces bisporus* yeasts..." (Claim 1, lines 1-2).

30 NOV 2001

09/926677

PCT Rule 34

CULTURE MEDIUM FOR THE DETECTION OF ZYGOSACCHAROMYCES

Object of the Invention

The present invention refers to a differential and selective culture medium containing glucose, formic acid and an acid-base indicator, for the detection in a sample, after 48 hours, of *Zygosaccharomyces bailii* and *Zygosaccharomyces bisporus* yeasts, two of the most dangerous species when considering food deterioration, and to a process for the detection of *Zygosaccharomyces bailii* and *Zygosaccharomyces bisporus* yeasts using the referred culture medium. Is a further object of the present invention the use of the referred culture medium in a gallery of yeasts identification tests.

State of the prior art

Yeasts are a growing problem in the food industry. The use of milder preservation processes in order to maintain the organoleptic properties of the product, of packages with modified atmospheres, and of new formulations, designed to avoid bacterial contamination are, nevertheless, favorable to yeast contamination. Although some pathogenic yeast species have been detected in food and the opportunistic strains may be dangerous to a fraction of the population, the fundamental risk of contamination that arises is not one of sanitary nature, but it consists in the spoilage effects that certain yeasts, such as *Zygosaccharomyces bailii* and *Zygosaccharomyces bisporus* have in food products, with the consequent economic losses involved.

Heretofore, the study of the yeast microflora present in the most diverse habitats (*e.g.* food, nature), comprises a first strain isolation stage, using the general selective yeast culture media, and a second identification stage of the isolated strains, through the use of conventional and/or molecular biology based methods. The classical yeast identification methods are based in a series of vegetative and sexual reproduction characteristics, and comprise a

large range of physiologic and biochemical tests. It is a demanding work that only produces results after at least one to two weeks, and requires a great deal of experience for the correct interpretation of the results. The molecular biology based methods are, generally, faster than the classical ones, but they also require a good amount of operator experience and involve expensive equipment and reactants.

There are some culture media commercially available for the detection of yeasts in wines, namely the Wallerstein Laboratory Nutrient Medium, WLN, used for detecting fermenting yeasts, and the Wallerstein Laboratory Differential Medium, WLD, which allow the detection of lactic and acetic bacteria as well as of yeasts belonging to the non-fermenting flora (both from Difco). However, these prior art media are not capable to differentiate the yeasts, particularly the *Zygosaccharomyces bailii* species.

There is therefore the necessity for a culture medium and a process for the detection and identification of *Zygosaccharomyces bailii* and *Zygosaccharomyces bisporus*, rapid and efficient, and which is thus an alternative means to the conventional techniques for the rapid detection of these species.

Description of the invention

It was surprisingly found that *Zygosaccharomyces bailii* and *Zygosaccharomyces bisporus* yeasts, when grown in a medium containing glucose, formic acid and an appropriated acid-base indicator, lead to a rapid change in the medium color and to the formation of colored colonies after 48 hours, these changes being characteristic and exclusive of the referred yeasts in the referred culture medium.

It was also found that the medium according to the invention is differential for the *Zygosaccharomyces bailii* and *Zygosaccharomyces bisporus* yeasts,

Further, the culture medium according to the present invention can be used to integrate galleries of identification of yeasts.

Brief Description of the Figures

Figure 1 is a photograph showing the response of several yeasts (*Z. bailii* ISA 1265 and *Z. bailii* IGC 3806: positive response; *T. delbrueckii* ISA 1229 and *I. Orientalis* IGC 3806: negative response) in a solid medium according to the present invention containing glucose (0.1% w/v) and formic acid (0.3% v/v) at the end of 96 hours of incubation at 30°C. The *Z. bailii* yeasts shown a positive response revealed by a blue coloring of the culture medium in the dish, while the negative responses are shown as a green coloring which did not change during the incubation.

Figure 2 is a photograph showing the response of several yeasts in a liquid medium according to the present invention containing glucose (0.1% w/v) and formic acid (0.3% w/v) at the end of 48 hours of incubation at 30°C. All the *Z. bailii* strains induced the medium to change color to blue, while all the others maintained the green color.

Figure 3 shows the morphology of *Zygosaccharomyces bailii* yeast colonies in a culture medium according to the present invention containing 0.3% (v/v) of formic acid and 0.1% (w/v) of glucose, obtained by the use of the method of membrane filtration, after 96 hours of incubation at the temperature of 30°C. The colonies can be observed well defined with a blue color

Figure 4 shows the morphology of *S. cerevisiae* and *Zygosaccharomyces bailii* yeast colonies in a culture medium according to the present invention containing 0.2% (v/v) of formic acid and 0.1% (w/v) of glucose, obtained by the use of the method of membrane filtration, after 96 hours of incubation at the temperature of 30°C. The *Z. bailii* colonies shown a blue coloring, perfectly distinct from the creme coloring of the other colonies.

Figure 5 shows the morphology of *P. membranaefaciens* and *Zygosaccharomyces bailii* yeast colonies in a culture medium according to the present invention containing 0.2% (v/v) of formic acid and 0.1% (w/v) of glucose, obtained by the use of the method of membrane filtration, after 96 hours of incubation at the temperature of 30°C. The *Z. bailii* colonies are totally distinguishable by its morphology and blue color.

Preferred embodiments of the invention

In a preferred embodiment of the present invention the differential and selective culture medium, for identification of *Zygosaccharomyces bailii* and *Zygosaccharomyces bisporus* yeasts in a sample, after 48 hours of incubation, comprises a base mineral medium, including bromocresol green as the acid-base indicator, supplemented with oligoelements and vitamins, 0.05% to 0.1% (w/v) of glucose and 0.1% to 0.5% (v/v) of formic acid as the only energy and carbon sources, and optionally agar and an inhibitor of bacterial growth.

In this embodiment of the invention, the bromocresol green provides the medium with a green coloring that will be converted into blue through the action of the *Zygosaccharomyces bailii* and *Zygosaccharomyces bisporus* yeasts during incubation under appropriate conditions. Additionally, the colonies of these yeasts will also present a blue color. The change of color of the culture medium is characteristic of these yeast species, as illustrated in examples 1 and 2, thus allowing the detection of the presence thereof in a sample only by the color changing.

The process according to the present invention will now, be illustrated by means of the non limitative examples below:

Examples

Example 1

This example illustrates the preparation of a solid culture medium according to the present invention and shows that it is effective in the identification of *Z. bailii* and *Z. bisporus* yeasts.

A culture medium is prepared comprising the following ingredients:

Table 1 **Culture medium composition for the detection of the *Zygosaccharomyces bailii* and *Zygosaccharomyces bisporus* yeasts**

Compound			Concentration (%)	
Base Medium	Ammonium sulphate	$(NH_4)_2SO_4$	0.5	(w/v)
	Potassium dihydrogenosulphate	KH_2PO_4	0.5	(w/v)
	Magnesium sulphate hetpahydrate	$MgSO_4 \cdot 7H_2O$	0.05	(w/v)
	Calcium chloride dihydrate	$CaCl_2 \cdot 2\ H_2O$	0.013	(w/v)
	Bromocresol green	$C_{21}H_{14}Br_4O_5S$	0.005	(w/v)
	Agar	-	2.0	(w/v)
Glucose	-	$C_6H_{12}O_6$	0.1	(w/v)
Formic acid	-	CH_2O_2	0.4	(v/v)
Oligoelements Solution A	(Composition according to Table 2)	-	0.05	(v/v)
Oligoelements Solution B	(Composition according to Table 2)	-	0.05	(v/v)
Vitamin Solution	(Composition according to Table 2)	-	0.05	(v/v)

Table 2 **Oligoelements and vitamin solutions composition**

Compound			Concentration (%)	
Oligoelements Solution A	Boric acid	H_3BO_3	1.0	(w/v)
	Potassium Iodide	KI	0.2	(w/v)
	Sodium molibdate dihydrate	$Na_2MoO_4 \cdot 2H_2O$	0.4	(w/v)
Oligoelements Solution B	Copper sulphate pentahydrate	$CuSO_4 \cdot 5H_2O$	0.08	(w/v)
	Iron chloride hexahydrate	$FeCl_3 \cdot 6\ H_2O$	0.4	(w/v)
	Manganese sulphate hexahydrate	$MnSO_4 \cdot 4H_2O$	0.8	(w/v)
	Tin sulphate hexahydrate	$ZnSO_4 \cdot 7H_2O$	0.8	(w/v)
	Hydrochloric acid	HCl 10^{-3}N	0.8	(v/v)
Vitamin Solution	Biotin	$C_{10}H_{16}N_2O_3S$	0.001	(w/v)
	Calcium panthotenate	$C_9H_{16}NO_5 \cdot 1/2\ Ca$	0.08	(w/v)
	Mioinositol	$C_6H_{12}O_6$	4.0	(w/v)
	Niacin	$C_6H_5NO_2$	0.16	(w/v)
	Pyridoxine hydrochloride	$C_8H_{11}NO_3 \cdot HCl$	0.16	(w/v)
	Thiamin hydrochloride	$C_{12}H_{17}ClN_4OS \cdot HCl$	0.16	(w/v)

The base medium compounds are dissolved in 4/5 of the estimated deionized water volume, and the sterilization is accomplished in autoclave at 121°C, for 20 minutes.

The other medium compounds (glucose, formic acid, oligoelements solution A, oligoelements solution B, and vitamin solution) are dissolved in the remaining water volume so that the final concentration of these compounds equals the values mentioned in Table 1. The pH must be adjusted to 4.5 with HCl 1M. The sterilization is accomplished by filtration. This solution and the base medium are annealed at 50±5°C before being mixed together. The whole medium is homogenized and dispensed into Petri dishes.

The yeast strains to be identified, previously purified and inoculated in agar slants with a generic yeast culture medium (yeast extract medium, peptone, and glucose), are incubated for 48 hours at 28°C. An loopful is transferred to the culture medium with glucose and formic acid, prepared above. The inoculation is made by streaking and the plates are incubated at 30°C, for a minimum time of 48 hours. Alternatively, the inoculation may be done with a cotton smear containing an equivalent biomass amount.

The results obtained are presented in Table 3.

Table 3 Inoculation by streaking - response of several yeasts in the culture medium containing glucose and formic acid (0.4% v/v) after 48 hours of incubation at 30°C.

Species	N. of tested strains	Culture medium color
Zygosaccharomyces bailii	15	blue
Zygosaccharomyces bisporus	5	blue
Zygosaccharomyces rouxii	3	blue*
Zygosaccharomyces florentinus	1	green
Saccharomyces bayanus	2	green
Saccharomyces cerevisiae	21	green
Saccharomyces pastorianus	2	green
Saccharomycodes ludwigii	3	green
Schizosaccharomyces pombe	4	green
Pichia membranaefaciens	13	green
Pichia anomala	7	green
Dekkera anomala	3	green
Dekkera bruxellensis	4	green
Debaryomyces hansenii	2	green
Issatchenkia orientalis	6	green
Kluyveromyces marxianus	5	green
Kloeckera apiculata	1	green
Lodderomyces elongisporus	2	green
Rhodotorula mucilaginosa	2	green
Torulaspora delbrueckii	7	green

* the change in the medium color was observed after an additional incubation period of 24-48 hours

A change in the culture medium color from green to blue was observed in all of the tested *Z. bailii* strains. However, it was observed that 3 of the 8 tested *Z. bisporus* strains converted the medium color only after an additional incubation period of 24 to 48 hours. For all the strains of the other species tested a negative result was observed, since the medium color did not change.

The results that were obtained show that the culture medium according to the present invention is suitable and effective for the detection of *Z. bailii* and *Z. bisporus* directly inoculated from cultures in solid medium after a minimum incubation period of 48 hours.

Example 2

The same procedure as Example 1 was used, differing only in that the inoculation was made with single strain cell suspensions instead of cells originated in solid medium. The cells are also originated from agar slants as disclosed in Example 1. The cell suspensions are prepared in deionized water in such a way that the optical density (OD_{640}) lies within the range of 0.7 to 1.0. 10 µl drops of these suspensions are placed on the surface of Petri dishes containing the medium disclosed in Example1. The plates were incubated at 30°C for 48 hours.

The results obtained are presented in Table 4. These results are similar to the ones presented for Example 1.

Table 4 **Application of cell suspensions on the surface of the solid medium - response of several yeasts in the culture medium containing glucose and formic acid (0.4% v/v) after 48 hours of incubation at 30°C.**

Species	N. of tested strains	Culture medium color
Zygosaccharomyces bailii	15	blue
Zygosaccharomyces bisporus	5	blue
Zygosaccharomyces rouxii	3	blue*
Zygosaccharomyces florentinus	1	green
Saccharomyces bayanus	2	green
Saccharomyces cerevisiae	21	green
Saccharomyces pastorianus	2	green
Saccharomycodes ludwigii	3	green
Schizosaccharomyces pombe	4	green
Pichia membranaefaciens	13	green
Pichia anomala	7	green
Dekkera anomala	3	green
Dekkera bruxellensis	4	green
Debaryomyces hansenii	2	green
Issatchenkia orientalis	6	green
Kluyveromyces marxianus	5	green
Kloeckera apiculata	1	green
Lodderomyces elongisporus	2	green
Rhodotorula mucilaginosa	2	green
Torulaspora delbrueckii	7	green

* the change in the medium color was observed after an additional incubation period of 72-96 hours

Table 5 Inoculation of cell suspensions in liquid medium - resp nse of s v ral yeasts in the culture medium containing glucose and formic acid (0.4% v/v) after 48 hours of incubation at 30°C.

Species	N. of tested strains	Culture medium color
Zygosaccharomyces bailii	15	blue
Zygosaccharomyces bisporus	5	blue
Zygosaccharomyces rouxii	3	blue*
Zygosaccharomyces florentinus	1	green
Saccharomyces bayanus	2	green
Saccharomyces cerevisiae	21	green
Saccharomyces pastorianus	2	green
Saccharomycodes ludwigii	3	green
Schizosaccharomyces pombe	4	green
Pichia membranaefaciens	13	green
Pichia anomala	7	green
Dekkera anomala	3	green
Dekkera bruxellensis	4	green
Debaryomyces hansenii	2	green
Issatchenkia orientalis	6	green
Kluyveromyces marxianus	5	green
Kloeckera apiculata	1	green
Lodderomyces elongisporus	2	green
Rhodotorula mucilaginosa	2	green
Torulaspora delbrueckii	7	green

* the change in the medium color was observed after an additional incubation period of 48-72 hours

The culture medium according to the present invention, in the liquid form, is equally suitable and effective for the detection of *Z. bailii* and *Z. bisporus* from pure culture suspensions after a minimum incubation period of 48 hours.

Example 4

This Example shows that the culture medium according to the present invention is selective for yeasts of the *Z. bailii* and *Z. bisporus* species in samples of mixed yeasts populations.

A similar procedure as in Example 3 is used, differing only in that the cell suspensions used are pure or mixed (in equal ratios) yeast cell suspensions, and in that the method of membrane filtration is used. The cell suspension is prepared as in Example 2. The mixed cultures are prepared from pure culture

suspensions. In this case, the inoculations are accomplished using an aliquot of the suitably diluted suspension that is filtered under vacuum through a sterilized filtration membrane (pores of 0.45 μm), the filters are then placed Petri dishes, and the dishes containing the filters on the surface of the medium disclosed in Example 1, are incubated at 30°C for 96 hours. As a reference culture medium (corresponding to a recovery ratio of 100%) a generic yeast culture medium is used (yeast extract medium, peptone, and glucose).

The results obtained are presented in Table 6. The recovery ratio of *Z. bailii* cells in the medium disclosed in Example 1 is about 60 to 70 %, regardless of the presence of other yeast species. The culture medium was shown to be highly selective since the recovery ratio of *S. cerevisiae, P. membranaefaciens* and *D. anomala* was significantly reduced, lower the 0.01%.

S. cerevisiae, P. membranaefaciens and *D. anomala* being representative examples of contaminant species in wines, the culture medium according to the invention will be useful and appropriate for the identification of *Z. bailii* in contaminated wines samples.

Table 6 **Recovery ratio (%) obtained by the method of membrane filtration after 96 hours of incubation at 30°C.**

Species	*Z. bailii* recovery ratio
Zygosaccharomyces bailii	65
Zygosaccharomyces bailii	57
Saccharomyces cerevisiae	n.d
Zygosaccharomyces bailii	67
Pichia membranaefaciens	n.d.
Dekkera anomala	n.d
Saccharomyces cerevisiae	< 0,002
Pichia membranaefaciens	0,011
Dekkera anomala	<0,004

n.d. not determined

The Figures 3, 4, and 5 show the colony morphology of different species in pure and mixed cultures, being remarkable the easy discrimination between the 3 species using only the colonies color and morphology.

In some cases non-typical colonies (*ca.* 2-3%) with a light blue coloring or with an intense blue coloring can be present. The first of these (Fig. 4), with a morphology similar to that of *S. cerevisiae*, were judged as belonging to this species. The light coloring of these colonies is due to the incorporation of the indicator after the color change induced by the presence of *Z. bailii*. The second kind of colonies (Fig. 5), with a similar morphology to that of *P. membranaefaciens* were judged as belonging to this species, the intense coloring being due to the high affinity of these cells for the indicator after the color changing induced by the presence of *Z. bailii*. This characteristic was equally observed for the pure cultures of *P. membranaefaciens* that showed a very intense green coloring in contrast with those of *S. cerevisiae*, that under these conditions, showed a green-cream coloring. However, the discrimination between those colonies is clear as can be seen in the appended Figures 4 and 5.

Example 5

This example shows the differential ability of the culture medium according to the present invention and the enumeration of *Z. bailii* cells in wine samples.

The enumeration of *Z. bailii* cells in wine samples is made using membrane filtration (according to the method disclosed in Example 4). For the determination of the number of colony forming units (CFU)/ml of wine is done after 96 hours of incubation at the temperature of 30°C. Other commercial culture media presently used for the detection of yeasts in wines (Wallerstein Laboratory Differential Medium, WLD, and Wallerstein Laboratory Nutrient Medium, WLN, both marketed by Difco) are tested in parallel. The WLN medium is used for the detection of fermenting yeasts, while the WLD

period of 48 hours. The same is valid for the detection of Z. bailii in a medium with 0,5% acid formic concentration.

Example 8

This example shows the effect of formic acid concentration in the culture medium according to the present invention on the medium selectivity.

The procedure of Example 4 was used, but using different concentrations of formic acid in the culture medium.

The results obtained are presented in Table 10. These results and the ones from Example 4 show that the recovery ratio of *Z. bailii* cells in the medium decreases with the increasing of the acid formic concentration, being independent of the presence of other yeast species such those that can be found in contaminated wines. For 2 of these other 3 tested species the recovery ratio also decreases with the increase in the formic acid concentration.

Table 10 **Recovery ratio (%) obtained by the method of membrane filtration after 96 hours of incubation at 30°C.**

Species	*Z. bailii* recovery ratio		
	formic acid 0.2%	formic acid 0.3%	formic acid 0.5%
Zygosaccharomyces bailii	82	78	42
Zygosaccharomyces bailii	82	81	35
Saccharomyces cerevisiae		n.d.	
Zygosaccharomyces bailii	99	94	34
Pichia membranaefaciens	n.d.	n.d.	n.d.
Dekkera anomala	n.d.	n.d.	n.d.
Saccharomyces cerevisiae	30	4	<0.002
Pichia membranaefaciens	55	5.9	<0.004
Dekkera anomala	<0.004	<0.004	<0.004

n.d. not determined

Thus, it was shown that the culture medium according to the present invention has characteristics of a selective and differential culture medium appropriated and highly effective for the detection, identification and

Claims

1. A differential and selective culture medium for *Zygosaccharomyces bailii* e *Zygosaccharomyces bisporus* yeasts, characterized in that it comprises a base mineral medium supplemented with vitamins, oligoelements, glucose and formic acid as the only carbon and energy sources, an appropriated acid-base indicator and, optionally an antibiotic inhibitor of bacterial growth and agar.

2. Culture medium according to claim 1 characterized in that glucose is present in a concentration from 0.05% to 0.1% (p/v), preferably 0.1% (p/v).

3. Culture medium according to claim 1 characterized in that formic acid is present in a concentration, dependent of the desired differentiability and selectivity, from 0.1% to 0.5% (v/v), preferably from 0.2% to 0.4% (v/v), and more preferably 0.4% (v/v).

4. Culture medium according to claim 3 characterized in that the formic acid concentration is preferably 0.4% (v/v).

5. Culture medium according to claim 1 characterized in that the base mineral medium comprises amonium sulphate (0.5% (w/v)), potassium dihidrogenosulphate (0.5% (w/v)), magnesium sulphate hetpahidrate (0.05% (w/v)) and calcium chloride dihydrate (0.013% (w/v)); the oligoelements solution A (0.05% (v/v)) comprises boric acid (1.0% (w/v)), potassium iodide (0.2% (w/v)) and sodium molibdate dihidrate (0.4% (w/v)); the oligoelements solution B (0.05% (v/v)) comprises copper sulphate pentahidrate (0.08% (w/v)), iron chloride hexahidrate (0.4% (w/v)), manganese sulphate hexahidrate (0.8% (w/v)), tin sulphate hexahidrate (0.8% (w/v)) and hydrochloric acid (HCl 10^{-3}N, 0.8% (v/v)); and the vitamin solution (0.05% (v/v)) comprises biotin (0.001% (w/v)), calcium panthotenate (0.08 % (w/v)),

mioinositol (4.0% (w/v)), niacin (0.16% (w/v)), pyridoxine hydrochloride (0.16% (w/v)) and thiamin hydrochloride (0.16% (w/v)).

6. Culture medium according to claim 1 characterized in that the acid-base indicator is one having a pK_i between 4.5 and 4.8, preferably bromocresol green.

7. Culture medium according to claim 6 characterized in that the pH is adjusted to 4.3-4.8, preferably 4.5.

8. Culture medium according to claim 1 characterized in that it further contains an antibiotic inhibitor of bacterial growth, in the usually used concentrations for this purpose, for use with mixed population samples containing bacteria.

9. A culture medium according to any previously claim characterized in that it contains all the ingredients except agar, that is in its liquid form.

10. A differential and selective culture medium for *Zygosaccharomyces bailii* e *Zygosaccharomyces bisporus* yeasts, characterized in that it is composed of

Glucose	0.1% (w/v)
Formic acid	0.4% (v/v)
Base Medium:	
Ammonium sulphate	0.5% (w/v)
Potassium dihydrogenosulphate	0.5% (w/v)
Magnesium sulphate hetpahydrate	0.05% (w/v)
Calcium chloride dihydrate	0.013% (w/v)
Bromocresol green	0.005% (w/v)
Agar	2.0% (w/v)
Oligoelements Solution A	0.05% (v/v)
Boric acid	1.0% (w/v)

Potassium Iodide	0.2% (w/v)
Sodium molibdate dihydrate	0.4% (w/v)
Oligoelements Solution B	0.05% (v/v)
Copper sulphate pentahydrate	0.08% (w/v)
Iron chloride hexahydrate	0.4% (w/v)
Manganese sulphate hexahydrate	0.8% (w/v)
Tin sulphate hexahydrate	0.8% (w/v)
Hydrochloric acid, HCl 10^{-3}N,	0.8% (v/v)
Vitamin Solution	0.05% (v/v)
Biotin	0.001% (w/v)
Calcium panthotenate	0.08% (w/v)
Mioinositol	4.0% (w/v)
Niacin	0.16% (w/v)
Pyridoxine hydrochloride	0.16% (w/v)
Thiamin hydrochloride	0.16% (w/v)

the pH being adjusted to pH 4.6 with HCl 1M.

11. Culture medium according to any previously claim characterized in that the medium is prepared by dissolving the base medium compounds in 4/5 of the estimated deionized water volume, the sterilization being accomplished in autoclave at 121°C, for 20 minutes, by dissolving the other medium compounds in the remaining water so that the final concentration of these compounds equals the desired values, the sterilization being accomplished by filtration, annealing this solution and the base medium at about 50±5°C, before mixing the same and to adjust the final pH value to the desired value.

12. Process for the detection of *Zygosaccharomyces bailii* e *Zygosaccharomyces bisporus* yeasts characterized by the use of a differential and selective culture medium for the referred yeast species, comprising a base mineral medium supplemented with vitamins, oligoelements, glucose and

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PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
84 810 - PCT		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/PT00/00004	31/05/2000	31/05/1999

International Patent Classification (IPC) or national classification and IPC
C12Q1/04

Applicant
UNIVERSIDADE DO MINHO et al.

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 7 sheets, including this cover sheet.

   ☒ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 16 sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☐ Priority
   - III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☐ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   - VI ☐ Certain documents cited
   - VII ☐ Certain defects in the international application
   - VIII ☒ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
15/12/2000	28.02.2001

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INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/PT00/00004

I. Basis of the report

1. This report has been drawn on the basis of *(substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments (Rules 70.16 and 70.17).)*:
Description, pages:

3-5,12,17,18,20	as originally filed
1,2,6-11,13-15,15a, 19	filed with the demand

Claims, No.:

12 (part),13-16	as originally filed
1-11,12 (part)	filed with the demand

Drawings, sheets:

1/2,2/2	as originally filed

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

   These elements were available or furnished to this Authority in the following language: , which is:

   - ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
   - ☐ the language of publication of the international application (under Rule 48.3(b)).
   - ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

   - ☐ contained in the international application in written form.
   - ☐ filed together with the international application in computer readable form.
   - ☐ furnished subsequently to this Authority in written form.
   - ☐ furnished subsequently to this Authority in computer readable form.
   - ☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
   - ☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

   ☐ the description, pages:

   ☐ the claims, Nos.:

   ☐ the drawings, sheets:

5. ☒ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

   (Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)
   see separate sheet

6. Additional observations, if necessary:
   see separate sheet

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	1-16
	No:	Claims	
Inventive step (IS)	Yes:	Claims	1-16
	No:	Claims	
Industrial applicability (IA)	Yes:	Claims	1-16
	No:	Claims	

2. Citations and explanations
   see separate sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:
see separate sheet

SECTION I

5. The amendments filed with the demand (explained in the accompanying letter dated 14.12.00) introduce subject-matter which extends beyond the content of the application as filed, contrary to Article 34(2)(b) PCT. The amendments concerned are the following:

   On page 10, Table 3; page 11, Table 4 and page 13, Table 5, a line has been inserted between the 3rd and 4th lines stating:

   "*Zygosaccharomyces rouxii* 6 green"

   No basis could be found for this amendment in the application as filed (i.e. part of the amendments referred to under point 7 of the aforementioned letter dated 14.12.00).

6. Additional observations

   Present Claims 1 to 12(part), filed with the demand, embrace amendments to original Claims 5 and 10 carried out under Art. 34 PCT, as well as amendments under Art. 19 PCT to original Claims 3, 5 and 10, which were filed with the letter dated 06.11.00.

SECTION V

2. CITATIONS AND EXPLANATIONS

2.1 The present application discloses a differential and selective culture medium suitable for the detection and identification of *Zygosaccharomyces bailii* and *Zygosaccharomyces bisporus* yeasts, which is considered to be neither anticipated nor suggested by the documents cited in the International Search

Report. The novel selective medium, which seems to be specially useful for detecting the aforementioned food deteriorating yeasts, comprises a base mineral medium supplemented with vitamins, oligoelements, glucose and formic acid as the only carbon and energy sources, and bromocresol green as an acid-base indicator having a pK_i between 4.5 and 4.8. This indicator provides the medium with a green color, that is converted to blue through the action of *Z. bailii* and *Z. bisporus* yeasts during incubation under appropriate conditions. As substantiated in the description (see Examples 1 and 2), when growing on solid medium, the colonies formed by *Z. bailii* and *Z. bisporus* will be easily distinguishable from colonies arising from other possible yeasts, because they present a blue color.

In line with the above present Claims 1-16 would appear to relate to subject-matter which is novel and inventive over the available prior art and thus satisfies the criteria set forth in Art. 33(2) and (3) PCT (however, see Section VIII below).

The subject-matter encompassed by Claims 1 to 16 is also susceptible of industrial applicability as required by Art. 33(4) PCT

SECTION VIII

1. It is clear from the description on page 3, lines 13-18 that the use of an acid-base indicator having a pK_i between 4.5 and 4.8 (particularly bromocresol green) is a feature essential to the performance of the invention.

Since independent Claims 1 and 12 do not contain this feature they do not meet the requirement following from Article 6 PCT taken in combination with Rule 6.3(b) PCT that any independent claim must contain all the technical features essential to the definition of the invention.

In connection with the foregoing objection it is noted that no appropriate acid-base indicator different from bromocresol green is mentioned in the present disclosure (see the supporting description on page 3, lines 15-17; page 4, lines 7-11; page 7, lines 13-14 and 19-26; Table 1 on page 8 and the Examples). Independent Claims

1 and 12 are therefore not supported by the description as required by Article 6 PCT, as their scope is broader than justified by the actual disclosure of the invention.

The above deficiency affects *mutatis mutandis* the definition of the intended subject-matter according to dependent Claims 2-5, 8-9, 11 and 14 and appended Claims 15 and 16, insofar as these claims rely either on the medium of Claim 1 or the process of Claim 12.

2. On page 4, lines 17-22 of the description the following is explained:

"The culture medium object of the invention is prepared by autoclave sterilization of the base mineral medium in deionized water. The medium is then allowed to cool, and before solidifying, the glucose, formic acid, oligoelements and vitamins, prepared as adequate solutions and previously sterilized, are added under aseptic conditions"

Considering the foregoing, the term "mixed", which is employed on present page 9, line 10 (instead of the previously used "annealed") seems to be inadequate. Probably the subject sentence instead of "... are *annealed at* ...", was meant to read, for instance, "The solution and the base medium are *brought to* ..."

It is also noted that present Claim 11 reads "... *annealing* this solution and the base medium at..." (but in line with the above should have better indicated, for example, "... *bringing* this solution and the base medium *to*...).

Moreover, it seems that the last line of Claim 11 should have read "before mixing the same and adjusting the final pH to the desired value"

3. The statement in the description on page 20, lines 13-16 implies that the subject-matter for which protection is sought may be different to that defined by the claims, thereby resulting in lack of clarity (Article 6 PCT) when used to interpret them (see also the PCT Guidelines, III-4.3a).

4. The following expressions appear to contain wording/clerical mistakes:

"... the filters are then placed Petri dishes..." (page 14, lines 3-4).

"... acid formic..." (page 18, line 9).

"... culture medium for *Zygosaccharomyces bailii* e *Zygosaccharomyces bisporus* yeasts..." (Claim 1, lines 1-2).

Culture medium for ~~the~~ detection of *Zygosaccharomyces* ~~*bailii* and *Zygosaccharomyces bisporus* Yeasts~~

Object of the Invention

The present invention refers to a differential and selective culture medium containing glucose, formic acid and an acid-base indicator, for the detection in a sample, after 48 hours, of *Zygosaccharomyces bailii* and *Zygosaccharomyces bisporus* yeasts, two of the most dangerous species when considering food deterioration, and to a process for the detection of *Zygosaccharomyces bailii* and *Zygosaccharomyces bisporus* yeasts using the referred culture medium. ~~It is~~ It is a further object of the present invention the use of the referred culture medium in a gallery of yeasts identification tests.

State of the prior art

Yeasts are a growing problem in the food industry. The use of milder preservation processes in order to maintain the organoleptic properties of the product, of packages with modified atmospheres, and of new formulations, designed to avoid bacterial contamination are, nevertheless, favorable to yeast contamination. Although some pathogenic yeast species have been detected in food and the opportunistic strains may be dangerous to a fraction of the population, the fundamental risk of contamination that arises is not one of sanitary nature, but it consists in the spoilage effects that certain yeasts, such as *Zygosaccharomyces bailii* and *Zygosaccharomyces bisporus* have in food products, with the consequent economic losses involved.

Heretofore, the study of the yeast microflora present in the most diverse habitats (*e.g.* food, nature), comprises a first strain isolation stage, using the general selective yeast culture media, and a second identification stage of the isolated strains, through the use of conventional and/or molecular biology based methods. The classical yeast identification methods are based in a series of vegetative and sexual reproduction characteristics, and comprise a

large range of physiologic and biochemical tests. It is a demanding work that only produces results after at least one to two weeks, and requires a great deal of experience for the correct interpretation of the results. The molecular biology based methods are, generally, faster than the classical ones, but they also require a good amount of operator experience and involve expensive equipment and reactants.

There are some culture media commercially available for the detection of yeasts in wines, namely the Wallerstein Laboratory Nutrient Medium, WLN, used for detecting fermenting yeasts, and the Wallerstein Laboratory Differential Medium, WLD, which allow the detection of lactic and acetic bacteria as well as of yeasts belonging to the non-fermenting flora (both from Difco). However, these prior art media are not capable to differentiate the yeasts, particularly the *Zygosaccharomyces bailii* species.

There is therefore the necessity for a culture medium and a process for the detection and identification of *Zygosaccharomyces bailii* and *Zygosaccharomyces bisporus*, rapid and efficient, and which is thus an alternative means to the conventional techniques for the rapid detection of these species.

Description of the invention

It was surprisingly found that *Zygosaccharomyces bailii* and *Zygosaccharomyces bisporus* yeasts, when grown in a medium containing glucose, formic acid and an appropriated acid-base indicator, lead to a rapid change in the medium color and to the formation of colored colonies after at least 48 hours, these changes being characteristic and exclusive of the referred yeasts in the referred culture medium.

It was also found that the medium according to the invention is differential for the *Zygosaccharomyces bailii* and *Zygosaccharomyces bisporus* yeasts,

Further, the culture medium according to the present invention can be used to integrate galleries of identification of yeasts.

Brief Description of the Figures

Figure 1 is a photograph showing the response of several yeasts (*Z. bailii* ISA 1265 and *Z. bailii* IGC ~~3806~~ 4806: positive response; *T. delbrueckii* ISA 1229 and *I. Orientalis* IGC 3806: negative response) in a solid medium according to the present invention containing glucose (0.1% w/v) and formic acid (0.3% v/v) at the end of 96 hours of incubation at 30°C. The *Z. bailii* yeasts shown a positive response revealed by a blue coloring of the culture medium in the dish, while the negative responses are shown as a green coloring which did not change during the incubation.

Figure 2 is a photograph showing the response of several yeasts in a liquid medium according to the present invention containing glucose (0.1% w/v) and formic acid (0.3% w/v) at the end of 48 hours of incubation at 30°C. All the *Z. bailii* strains induced the medium to change color to blue, while all the others maintained the green color.

Figure 3 shows the morphology of *Zygosaccharomyces bailii* yeast colonies in a culture medium according to the present invention containing 0.3% (v/v) of formic acid and 0.1% (w/v) of glucose, obtained by the use of the method of membrane filtration, after 96 hours of incubation at the temperature of 30°C. The colonies can be observed well defined with a blue color.

Figure 4 shows the morphology of *S. cerevisiae* and *Zygosaccharomyces bailii* yeast colonies in a culture medium according to the present invention containing 0.2% (v/v) of formic acid and 0.1% (w/v) of glucose, obtained by the use of the method of membrane filtration, after 96 hours of incubation at the temperature of 30°C. The *Z. bailii* colonies shown a blue coloring, perfectly distinct from the creme coloring of the other colonies.

Figure 5 shows the morphology of *P. membranaefaciens* and *Zygosaccharomyces bailii* yeast colonies in a culture medium according to the present invention containing 0.2% (v/v) of formic acid and 0.1% (w/v) of glucose, obtained by the use of the method of membrane filtration, after 96 hours of incubation at the temperature of 30°C. The *Z. bailii* colonies are totally distinguishable by its morphology and blue color.

Preferred embodiments of the invention

In a preferred embodiment of the present invention the differential and selective culture medium, for identification of *Zygosaccharomyces bailii* and *Zygosaccharomyces bisporus* yeasts in a sample, after at least 48 hours of incubation, comprises a base mineral medium, including bromocresol green as the acid-base indicator, supplemented with oligoelements and vitamins, 0.05% to 0.1% (w/v) of glucose and 0.1% to 0.5% (v/v) of formic acid as the only energy and carbon sources, and optionally agar and an inhibitor of bacterial growth.

In this embodiment of the invention, the bromocresol green provides the medium with a green coloring that will be converted into blue through the action of the *Zygosaccharomyces bailii* and *Zygosaccharomyces bisporus* yeasts during incubation under appropriate conditions. Additionally, the colonies of these yeasts will also present a blue color. The change of color of the culture medium is characteristic of these yeast species, as illustrated in examples 1 and 2, thus allowing the detection of the presence thereof in a sample only by the color changing.

The process according to the present invention will now, be illustrated by means of the non limitative examples below:

Examples

Example 1

This example illustrates the preparation of a solid culture medium according to the present invention and shows that it is effective in the identification of *Z. bailii* and *Z. bisporus* yeasts.

A culture medium is prepared comprising the following ingredients:

Table 1 **Culture medium composition for the detection of the *Zygosaccharomyces bailii* and *Zygosaccharomyces bisporus* yeasts**

Compound			Concentration (%)	
Base Medium	Ammonium sulphate	$(NH_4)_2SO_4$	0.5	(w/v)
	Potassium dihydrogen ~~sulphate~~ phosphate	KH_2PO_4	0.5	(w/v)
	Magnesium sulphate heptahydrate	$MgSO_4 \cdot 7H_2O$	0.05	(w/v)
	Calcium chloride dihydrate	$CaCl_2 \cdot 2\ H_2O$	0.013	(w/v)
	Bromocresol green	$C_{21}H_{14}Br_4O_5S$	0.005	(w/v)
	Agar	-	2.0	(w/v)
Glucose	-	$C_6H_{12}O_6$	0.1	(w/v)
Formic acid	-	CH_2O_2	0.4	(v/v)
Oligoelements Solution A	(Composition according to Table 2)	-	0.05	(v/v)
Oligoelements Solution B	(Composition according to Table 2)	-	0.05	(v/v)
Vitamin Solution	(Composition according to Table 2)	-	0.05	(v/v)

Table 2 **Oligoelements and vitamin solutions composition**

Compound			Concentration (%)	
Oligoelements Solution A	Boric acid	H_3BO_3	1.0	(w/v)
	Potassium Iodide	KI	0.2	(w/v)
	Sodium molibdate dihydrate	$Na_2MoO_4 \cdot 2H_2O$	0.4	(w/v)
Oligoelements Solution B	Copper sulphate pentahydrate	$CuSO_4 \cdot 5H_2O$	0.08	(w/v)
	Iron chloride hexahydrate	$FeCl_3 \cdot 6\ H_2O$	0.4	(w/v)
	Manganese sulphate ~~hexa~~ tetra hydrate	$MnSO_4 \cdot 4H_2O$	0.8	(w/v)
	Zinc ~~Zinq~~ sulphate ~~hexa~~ hepta hydrate	$ZnSO_4 \cdot 7H_2O$	0.8	(w/v)
	Hydrochloric acid	HCl 10^{-3}N	0.8	(v/v)
Vitamin Solution	Biotin	$C_{10}H_{16}N_2O_3S$	0.001	(w/v)
	Calcium panthotenate	$C_9H_{16}NO_5 \cdot 1/2\ Ca$	0.08	(w/v)
	Mioinositol	$C_6H_{12}O_6$	4.0	(w/v)
	Niacin	$C_6H_5NO_2$	0.16	(w/v)
	Pyridoxine hydrochloride	$C_8H_{11}NO_3 \cdot HCl$	0.16	(w/v)
	Thiamin hydrochloride	$C_{12}H_{17}ClN_4OS \cdot HCl$	0.16	(w/v)

The base medium compounds are dissolved in 4/5 of the estimated deionized water volume, and the sterilization is accomplished in autoclave at 121°C, for 20 minutes.

The other medium compounds (glucose, formic acid, oligoelements solution A, oligoelements solution B, and vitamin solution) are dissolved in the remaining water volume so that the final concentration of these compounds equals the values mentioned in Table 1. The pH must be adjusted to 4.5 with HCl 1M. The sterilization is accomplished by filtration. This solution and the base medium are ~~annealed~~ mixed at 50 ± 5°C before being mixed together. The whole medium is homogenized and dispensed into Petri dishes.

The yeast strains to be identified, previously purified and inoculated in agar slants with a generic yeast culture medium (yeast extract medium, peptone, and glucose), are incubated for 48 hours at 28°C. An loopful is transferred to the culture medium with glucose and formic acid, prepared above. The inoculation is made by streaking and the plates are incubated at 30°C, for a minimum time of 48 hours. Alternatively, the inoculation may be done with a cotton smear containing an equivalent biomass amount.

The results obtained are presented in Table 3. Typical responses of the below mentioned yeasts are shown in Figure 1.

Table 3 **Inoculation by streaking - response of sev ral yeasts in th culture medium containing glucose and formic acid (0.4% v/v) after 48 hours of incubation at 30°C.**

Species	N. of tested strains	Culture medium color
Zygosaccharomyces bailii	15	blue
Zygosaccharomyces bisporus	5	blue
Zygosaccharomyces ~~rouxii~~ bisporus	3	blue *
Zygosaccharomyces florentinus	1	green
Saccharomyces bayanus	2	green
Saccharomyces cerevisiae	21	green
Saccharomyces pastorianus	2	green
Saccharomycodes ludwigii	3	green
Schizosaccharomyces pombe	4	green
Pichia membranaefaciens	13	green
Pichia anomala	7	green
Dekkera anomala	3	green
Dekkera bruxellensis	4	green
Debaryomyces hansenii	2	green
Issatchenkia orientalis	6	green
Kluyveromyces marxianus	5	green
Kloeckera apiculata	1	green
Lodderomyces elongisporus	2	green
Rhodotorula mucilaginosa	2	green
Torulaspora delbrueckii	7	green

* the change in the medium color was observed after an additional incubation period of 24-48 hours

A change in the culture medium color from green to blue was observed in all of the tested *Z. bailii* strains. However, it was observed that 3 of the 8 tested *Z. bisporus* strains converted the medium color only after an additional incubation period of 24 to 48 hours. For all the strains of the other species tested a negative result was observed, since the medium color did not change.

The results that were obtained show that the culture medium according to the present invention is suitable and effective for the detection of *Z. bailii* and *Z. bisporus* directly inoculated from cultures in solid medium after a minimum incubation period of 48 hours.

(*) Zygosaccharomyces rouxii 6 green

Example 2

The same procedure as Example 1 was used, differing only in that the inoculation was made with single strain cell suspensions instead of cells originated in solid medium. The cells are also originated from agar slants as disclosed in Example 1. The cell suspensions are prepared in deionized water in such a way that the optical density (OD_{640}) lies within the range of 0.7 to 1.0. 10 µl drops of these suspensions are placed on the surface of Petri dishes containing the medium disclosed in Example1. The plates were incubated at 30°C for 48 hours.

The results obtained are presented in Table 4. These results are identical ~~similar~~ to the ones presented for Example 1.

Table 4 **Application of cell suspensions on the surface of the solid medium - response of several yeasts in the culture medium containing glucose and formic acid (0.4% v/v) after 48 hours of incubation at 30°C.**

Species	N. of tested strains	Culture medium color
Zygosaccharomyces bailii	15	blue
Zygosaccharomyces bisporus	5	blue
Zygosaccharomyces ~~rouxii~~ bisporus	3	blue*
Zygosaccharomyces florentinus	1	green
Saccharomyces bayanus	2	green
Saccharomyces cerevisiae	21	green
Saccharomyces pastorianus	2	green
Saccharomycodes ludwigii	3	green
Schizosaccharomyces pombe	4	green
Pichia membranaefaciens	13	green
Pichia anomala	7	green
Dekkera anomala	3	green
Dekkera bruxellensis	4	green
Debaryomyces hansenii	2	green
Issatchenkia orientalis	6	green
Kluyveromyces marxianus	5	green
Kloeckera apiculata	1	green
Lodderomyces elongisporus	2	green
Rhodotorula mucilaginosa	2	green
Torulaspora delbrueckii	7	green

* the change in the medium color was observed after an additional incubation period of 72-96 hours

(*) (see page 10)

Table 5 **Inoculation of cell suspensions in liquid m dium - response of s veral yeasts in the culture medium containing glucose and formic acid (0.4% v/v) after 48 hours of incubation at 30°C.**

Species	N. of tested strains	Culture medium color
Zygosaccharomyces bailii	15	blue
Zygosaccharomyces bisporus	5	blue
Zygosaccharomyces ~~rouxii~~ bisporus	3	blue*
Zygosaccharomyces florentinus	1	green
Saccharomyces bayanus	2	green
Saccharomyces cerevisiae	21	green
Saccharomyces pastorianus	2	green
Saccharomycodes ludwigii	3	green
Schizosaccharomyces pombe	4	green
Pichia membranaefaciens	13	green
Pichia anomala	7	green
Dekkera anomala	3	green
Dekkera bruxellensis	4	green
Debaryomyces hansenii	2	green
Issatchenkia orientalis	6	green
Kluyveromyces marxianus	5	green
Kloeckera apiculata	1	green
Lodderomyces elongisporus	2	green
Rhodotorula mucilaginosa	2	green
Torulaspora delbrueckii	7	green

* the change in the medium color was observed after an additional incubation period of 48-72 hours

The culture medium according to the present invention, in the liquid form, is equally suitable and effective for the detection of *Z. bailii* and *Z. bisporus* from pure culture suspensions after a minimum incubation period of 48 hours.

Example 4

This Example shows that the culture medium according to the present invention is selective for yeasts of the *Z. bailii* and *Z. bisporus* species in samples of mixed yeasts populations.

A similar procedure as in Example 3 is used, differing only in that the cell suspensions used are pure or mixed (in equal ratios) yeast cell suspensions, and in that the method of membrane filtration is used. The cell suspension is prepared as in Example 2. The mixed cultures are prepared from pure culture

suspensions. In this case, the inoculations are accomplished using an aliquot of the suitably diluted suspension that is filtered under vacuum through a sterilized filtration membrane (pores of 0.45 µm), the filters are then placed Petri dishes, and the dishes containing the filters on the surface of the medium disclosed in Example 1, are incubated at 30°C for 96 hours. As a reference culture medium (corresponding to a recovery ratio of 100%) a generic yeast culture medium is used (yeast extract ~~medium~~, peptone, and glucose). medium containing

The results obtained are presented in Table 6. The recovery ratio of *Z. bailii* cells in the medium disclosed in Example 1 is about 60 to 70 %, regardless of the presence of other yeast species. The culture medium was shown to be highly selective since the recovery ratio of *S. cerevisiae, P. membranaefaciens* and *D. anomala* was significantly reduced, lower the 0.01%.

S. cerevisiae, P. membranaefaciens and *D. anomala* being representative examples of contaminant species in wines, the culture medium according to the invention will be useful and appropriate for the identification of *Z. bailii* in contaminated wines samples.

Table 6 **Recovery ratio (%) obtained by the method of membrane filtration after 96 hours of incubation at 30°C.**

Species	*Z. bailii* recovery ratio
Zygosaccharomyces bailii	65
Zygosaccharomyces bailii	57
Saccharomyces cerevisiae	n.d
Zygosaccharomyces bailii	67
Pichia membranaefaciens	n.d.
Dekkera anomala	n.d
Saccharomyces cerevisiae	< 0,002
Pichia membranaefaciens	0,011
Dekkera anomala	<0,004

n.d. not determined

The Figures 3, 4, and 5 show the colony morphology of different species in pure and mixed cultures, being remarkable the easy discrimination between the 3 species using only the colonies color and morphology.

~~In some cases non typical colonies (*ca.* 2-3%) with a light blue coloring or with an intense blue coloring can be present. The first of these (Fig. 4), with a morphology similar to that of *S. cerevisiae*, were judged as belonging to this species. The light coloring of these colonies is due to the incorporation of the indicator after the color change induced by the presence of *Z. bailii*. The second kind of colonies (Fig. 5), with a similar morphology to that of *P. membranaefaciens* were judged as belonging to this species, the intense coloring being due to the high affinity of these cells for the indicator after the color changing induced by the presence of *Z. bailii*. This characteristic was equally observed for the pure cultures of *P. membranaefaciens* that showed a very intense green coloring in contrast with those of *S. cerevisiae*, that under these conditions, showed a green cream coloring. However, the discrimination between these colonies is clear as can be seen in the appended Figures 4 and 5.~~

< insert page 15a >

Example 5

This example shows the differential ability of the culture medium according to the present invention and the enumeration of *Z. bailii* cells in wine samples.

The enumeration of *Z. bailii* cells in wine samples is made using membrane filtration (according to the method disclosed in Example 4). For the determination of the number of colony forming units (CFU)/ml of wine is done after 96 hours of incubation at the temperature of 30°C. Other commercial culture media presently used for the detection of yeasts in wines (Wallerstein Laboratory Differential Medium, WLD, and Wallerstein Laboratory Nutrient Medium, WLN, both marketed by Difco) are tested in parallel. The WLN medium is used for the detection of fermenting yeasts, while the WLD

new page >

In mixed cultures of *S. cerevisiae* and *Z. bailii*, a few colonies (*ca.* 2-3%) with a light blue coloring and with a morphology similar to that of *S. cerevisiae* can be present, and that were judged as belonging to this species. The light coloring is due to the affinity of these cells for the indicator after the color changing induced by the presence of *Z. bailii*.

In mixed cultures of *P. membranaefaciens* and *Z. bailii,* an intense blue coloring of the colonies formed by *P. membranaefaciens* was observed. This characteristic is equally due to the high affinity of these cells for the indicator after the color changing induced by the presence of *Z. bailii*. However, the discrimination between those colonies is clear as can be seen in the appended Figure 5.

period of 48 hours. The same is valid for the detection of Z. bailii in a medium with 0,5% acid formic concentration.

Example 8

This example shows the effect of formic acid concentration in the culture medium according to the present invention on the medium selectivity.

The procedure of Example 4 was used, but using different concentrations of formic acid in the culture medium.

The results obtained are presented in Table 10. These results and the ones from Example 4 show that the recovery ratio of *Z. bailii* cells in the medium decreases with the increasing of the acid formic concentration, being independent of the presence of other yeast species such those that can be found in contaminated wines. For 2 of these other 3 tested species the recovery ratio also decreases with the increase in the formic acid concentration.

Table 10 **Recovery ratio (%) obtained by the method of membrane filtration after 96 hours of incubation at 30°C.**

Species	*Z. bailii* recovery ratio		
	formic acid 0.2%	formic acid 0.3%	formic acid 0.5%
Zygosaccharomyces bailii	82	78	42
Zygosaccharomyces bailii	82	81	35
Saccharomyces cerevisiae	n.d.	n.d.	n.d.
Zygosaccharomyces bailii	99	94	34
Pichia membranaefaciens	n.d.	n.d.	n.d.
Dekkera anomala	n.d.	n.d.	n.d.
Saccharomyces cerevisiae	30	4	<0.002
Pichia membranaefaciens	55	5.9	<0.004
Dekkera anomala	<0.004	<0.004	<0.004

n.d. not determined

Thus, it was shown that the culture medium according to the present invention has characteristics of a selective and differential culture medium appropriated and highly effective for the detection, identification and

Claims

1. A differential and selective culture medium for *Zygosaccharomyces bailii* e *Zygosaccharomyces bisporus* yeasts, characterized in that it comprises a base mineral medium supplemented with vitamins, oligoelements, glucose and formic acid as the only carbon and energy sources, an appropriated acid-base indicator and, optionally an antibiotic inhibitor of bacterial growth and agar.

2. Culture medium according to claim 1 characterized in that glucose is present in a concentration from 0.05% to 0.1% (p/v), preferably 0.1% (p/v).

3. Culture medium according to claim 1 characterized in that formic acid is present in a concentration, dependent of the desired differentiability and selectivity, from 0.1% to 0.5% (v/v), preferably from 0.2% to 0.4% (v/v).

4. Culture medium according to claim 3 characterized in that the formic acid concentration is preferably 0.4% (v/v).

5. Culture medium according to claim 1 characterized in that the base mineral medium comprises amonium sulphate (0.5% (w/v)), potassium dihydrogen ~~sulphate~~ phosphate (0.5% (w/v)), magnesium sulphate ~~hetpa~~hepta hydrate (0.05% (w/v)) and calcium chloride dihydrate (0.013% (w/v)); the oligoelements solution A (0.05% (v/v)) comprises boric acid (1.0% (w/v)), potassium iodide (0.2% (w/v)) and sodium molibdate dihydrate (0.4% (w/v)); the oligoelements solution B (0.05% (v/v)) comprises copper sulphate pentahydrate (0.08% (w/v)), iron chloride hexahydrate (0.4% (w/v)), manganese sulphate tetrahydrate (0.8% (w/v)), ~~tin~~ zinc sulphate heptahydrate (0.8% (w/v)) and hydrochloric acid (HCl 10^{-3}N, 0.8% (v/v)); and the vitamin solution (0.05% (v/v)) comprises biotin (0.001% (w/v)), calcium panthotenate (0.08% (w/v)),

mioinositol (4.0% (w/v)), niacin (0.16% (w/v)), pyridoxine hydrochloride (0.16% (w/v)) and thiamin hydrochloride (0.16% (w/v)).

6. Culture medium according to claim 1 characterized in that the acid-base indicator is one having a pK_i between 4.5 and 4.8, preferably bromocresol green.

7. Culture medium according to claim 6 characterized in that the pH is adjusted to 4.3-4.8, preferably 4.5.

8. Culture medium according to claim 1 characterized in that it further contains an antibiotic inhibitor of bacterial growth, in the usually used concentrations for this purpose, for use with mixed population samples containing bacteria.

9. A culture medium according to any previously claim characterized in that it contains all the ingredients except agar, that is in its liquid form.

10. A differential and selective culture medium for *Zygosaccharomyces bailii* e *Zygosaccharomyces bisporus* yeasts, characterized in that it is composed of

Glucose	0.1% (w/v)
Formic acid	0.4% (v/v)
Base Medium:	
Ammonium sulphate	0.5% (w/v)
Potassium dihydrogen ~~sulphate~~ phosphate	0.5% (w/v)
Magnesium sulphate ~~hetpa~~ hepta hydrate	0.05% (w/v)
Calcium chloride dihydrate	0.013% (w/v)
Bromocresol green	0.005% (w/v)
Agar	2.0% (w/v)
Oligoelements Solution A	0.05% (v/v)
Boric acid	1.0% (w/v)

Potassium Iodide	0.2% (w/v)
Sodium molibdate dihydrate	0.4% (w/v)
Oligoelements Solution B	0.05% (v/v)
Copper sulphate pentahydrate	0.08% (w/v)
Iron chloride hexahydrate	0.4% (w/v)
~~Manganese sulphate tetrahydrate~~	~~0.8% (w/v)~~
Zinc ~~Tin~~ sulphate heptahydrate	0.8% (w/v)
Hydrochloric acid, HCl 10^{-3}N,	0.8% (v/v)
Vitamin Solution	0.05% (v/v)
Biotin	0.001% (w/v)
Calcium panthotenate	0.08% (w/v)
Mioinositol	4.0% (w/v)
Niacin	0.16% (w/v)
Pyridoxine hydrochloride	0.16% (w/v)
Thiamin hydrochloride	0.16% (w/v)

the pH being adjusted to pH 4.~~6~~ 5 with HCl 1M.

11. Culture medium according to any previously claim characterized in that the medium is prepared by dissolving the base medium compounds in 4/5 of the estimated deionized water volume, the sterilization being accomplished in autoclave at 121°C, for 20 minutes, by dissolving the other medium compounds in the remaining water so that the final concentration of these compounds equals the desired values, the sterilization being accomplished by filtration, annealing this solution and the base medium at about 50 ± 5°C, before mixing the same and to adjust the final pH value to the desired value.

12. Process for the detection of *Zygosaccharomyces bailii* e *Zygosaccharomyces bisporus* yeasts characterized by the use of a differential and selective culture medium for the referred yeast species, comprising a base mineral medium supplemented with vitamins, oligoelements, glucose and

INTERNATIONAL SEARCH REPORT

Inte [illegible] Application No
PCT/[illegible]0/00004

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12Q1/04

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	CHEMICAL ABSTRACTS, vol. 133, no. 3, 17 July 2000 (2000-07-17) Columbus, Ohio, US; abstract no. 29819, M.-I. DE SILONIZ ET AL.: "Advances in the development of a methodology to identify common yeast contaminants of high sugar food products" page 449; column 1; XP002148559 abstract & FOOD TECHNOL. BIOTECHNOL., vol. 37, no. 4, 1999, pages 277-280, --- -/--	1-16

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
27 September 2000	11/10/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Griffith, G

INTERNATIONAL SEARCH REPORT

Inte[illegible]onal Application No
PCT/P[illegible]/00004

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	CHEMICAL ABSTRACTS, vol. 125, no. 9, 26 August 1996 (1996-08-26) Columbus, Ohio, US; abstract no. 113126, P. CERRUTTI ET AL.: "Inhibitory effects of vanillin on some food spoilage yeasts in laboratory media and fruit purees" page 1030; column 1; XP002148560 abstract & INT. J. FOOD MICROBIOL., vol. 29, no. 2,3, 1996, pages 379-386, ---	1-16
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INTERNATIONAL SEARCH REPORT

Inte [illegible] Application No
PCT/P[illegible]0/00004

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	CHEMICAL ABSTRACTS, vol. 100, no. 9, 27 February 1984 (1984-02-27) Columbus, Ohio, US; abstract no. 66580, W. BABEL ET AL.: "Improvement of growth yield of yeast on glucose to the maximum by using an additional energy source" page 468; column 1; XP002148563 abstract & ARCH. MICROBIOL., vol. 136, no. 3, 1983, pages 203-208,	

INTERNATIONAL SEARCH REPORT

Information on patent family members

Int[illegible] Application No
PCT/[illegible]0/00004

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		US 5989853 A	23-11-1999

09/926677
JC13 Rec'd PCT/PTO 30 NOV 2001

THE FOLLOWING IS THE ENGLISH TRANSLATION OF THE AMENDMENTS TO THE CLAIMS OF THE INTERNATIONAL APPLICATION UNDER PCT ARTICLE 19: AMENDED SHEETS (Pages 25 and 26).

AMENDED CLAIMS
[received by the International Bureau on 8 November 2000 (08.11.00);
original claims 3, 5 and 10 amended; remaining claims unchanged (2 pages)]

1. A differ ntial and selective culture medium for *Zygosaccharomyces bailii* e *Zygosaccharomyces bisporus* yeasts, characterized in that it comprises a base mineral medium supplemented with vitamins, oligoelements, glucose and formic acid as the only carbon and energy sources, an appropriated acid-base indicator and, optionally an antibiotic inhibitor of bacterial growth and agar.

2. Culture medium according to claim 1 characterized in that glucose is present in a concentration from 0.05% to 0.1% (p/v), preferably 0.1% (p/v).

3. Culture medium according to claim 1 characterized in that formic acid is present in a concentration, dependent of the desired differentiability and selectivity, from 0.1% to 0.5% (v/v), preferably from 0.2% to 0.4% (v/v).

4. Culture medium according to claim 3 characterized in that the formic acid concentration is preferably 0.4% (v/v).

5. Culture medium according to claim 1 characterized in that the base mineral medium comprises amonium sulphate (0.5% (w/v)), potassium dihidrogenosulphate (0.5% (w/v)), magnesium sulphate hetpahydrate (0.05% (w/v)) and calcium chloride dihydrate (0.013% (w/v)); the oligoelements solution A (0.05% (v/v)) comprises boric acid (1.0% (w/v)), potassium iodide (0.2% (w/v)) and sodium molibdate dihydrate (0.4% (w/v)); the oligoelements solution B (0.05% (v/v)) comprises copper sulphate pentahydrate (0.08% (w/v)), iron chloride hexahydrate (0.4% (w/v)), manganese sulphate tetrahydrate (0.8% (w/v)), tin sulphate heptahydrate (0.8% (w/v)) and hydrochloric acid (HCl 10^{-3}N, 0.8% (v/v)); and th vitamin solution (0.05% (v/v)) comprises biotin (0.001% (w/v)), calcium panthotenate (0.08% (w/v)),

	Potassium Iodide	0.2% (w/v)
	Sodium molibdate dihydrate	0.4% (w/v)
Oligoelements Solution B		0.05% (v/v)
	Copper sulphate pentahydrate	0.08% (w/v)
	Iron chloride hexahydrate	0.4% (w/v)
	Manganese sulphate tetrahydrate	0.8% (w/v)
	Tin sulphate heptahydrate	0.8% (w/v)
	Hydrochloric acid, HCl 10^{-3}N,	0.8% (v/v)
Vitamin Solution		0.05% (v/v)
	Biotin	0.001% (w/v)
	Calcium panthotenate	0.08% (w/v)
	Mioinositol	4.0% (w/v)
	Niacin	0.16% (w/v)
	Pyridoxine hydrochloride	0.16% (w/v)
	Thiamin hydrochloride	0.16% (w/v)

the pH being adjusted to pH 4.6 with HCl 1M.

11. Culture medium according to any previously claim characterized in that the medium is prepared by dissolving the base medium compounds in 4/5 of the estimated deionized water volume, the sterilization being accomplished in autoclave at 121°C, for 20 minutes, by dissolving the other medium compounds in the remaining water so that the final concentration of these compounds equals the desired values, the sterilization being accomplished by filtration, annealing this solution and the base medium at about 50±5°C, before mixing the same and to adjust the final pH value to the desired value.

12. Process for the detection of *Zygosaccharomyces bailii* e *Zygosaccharomyces bisporus* yeasts characterized by the use of a differential and selective culture medium for the referred yeast species, comprising a base mineral medium supplemented with vitamins, oligoelements, glucos and

DO/US WORKSHEET

U.S. Appl. No. 09/[redacted]26677 International Appl No. PT00/00004

Application filed by: ☐ 20 months ☑ 30 months

INTERNATIONAL APPLICATION PAPERS IN THE APPLICATION FILE:

- ☑ International application (RECORD COPY)
- ☐ Article 19 amendments
- ☑ PCT/IB/331
- ☑ PCT/IPEA/409 IPER (PCT/IPEA/416 on front)
- ☑ Annexes to 409
- ☑ Priority document(s) No. 1
- ☐ INTERNATIONAL APPLICATION ON DOUBLE SIDED PAPER (COPIES MADE)
- ☐ Request form PCT/RO/101
- ☐ PCT/IB/302
- ☑ PCT/ISA/210-Search Report
- ☑ Search Report references
- ☑ Other 306

RECEIPTS FROM THE APPLICANT: (other than checked above)

- ☑ Basic National Fee (paid or authorized to charge)
- Translation of international application as filed:
  - ☑ Description
  - ☑ Claims
  - ☑ Words in the drawing figure(s)
  - ☐ Article 19 amendments
  - ☑ Annexes to 409 Entered.
- ☐ Oath / Declaration
- ☐ DNA diskette
- ☑ Preliminary amendment(s) filed
- ☑ Information Disclosure Statement
- ☐ Assignment document
- ☐ Power of attorney/Change of address
- ☐ Substitute specification
- ☐ Verified small status claim
- ☐ Other

Notes: Use IA from IB.

35 U.S.C. 371 - Receipt of Request (PTO-1390)	30 NOV 2001
Date acceptable oath / declaration received	11 MAR 2002
Date complete 35 U.S.C 371 requirements met	"
102(e) Date	"
Date of completion of DO/EO 906 - Notification of Missing 102(e) Requirements	
Date of completion of DO/EO 907 - Notification of Acceptance for 102(e) date	
Date of completion of DO/EO 911 - Application accepted under 35 U.S.C. 1.11	
Date of completion of DO/EO 905 - Notification of Missing Requirements	10 Jan '02
Date of completion of DO/EO 916 - Notification of Defective Response	
Date of completion of DO/EO 903 - Notification of Acceptance	24 MAR 2002.
Date of completion of DO/EO 909 - Notification of Abandonment	

WIPO Publication

Publication No. WO 00/73494 A1

Publication Date 7 DEC 00

Publication Language English

Not Published
- ☐ U.S. only
- Designated
- ☑ EP request

Screening done by: SA

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May 1997